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Comparison of primers for the detection of *Salmonella enterica* serovars using real-time PCR

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ABSTRACT

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Aims: To evaluate the specificity and sensitivity of PCR primers for the detection of *Salmonella enterica* in a real-time PCR assay using pure cultures.

Methods and Results: Unenriched whole cells in sterile water were used as template for each PCR. SYBR Green dye was used for the nonspecific detection of dsDNA. The real-time PCR detection limits of five previously published primer sets used in conventional PCR applications were not below 3×10^3 CFU per reaction (rxn). A new primer set, Sen, was designed, which detected *Salm. enterica* Newport down to 6 CFU rxn^{-1} in one case, and gave an average detection limit of 35 CFU rxn^{-1} over three separate runs.

Conclusions: Primers originally designed for end-point PCR did not have adequate specificity or sensitivity compared with those specifically designed for real-time PCR.

Significance and Impact of the Study: This study emphasizes the importance of evaluating real-time PCR primer sets in pure cultures prior to testing in field samples. This study will benefit other researchers in selecting an appropriate primer set for real-time PCR detection of *Salm. enterica*.

Keywords: dissociation analysis, *invA* gene, primer dimer, SYBR Green, threshold cycle.

INTRODUCTION

Real-time PCR offers advantages over traditional PCR in that it is possible to watch the PCR reaction as it occurs, the PCR product does not have to be removed from its reaction chamber for post-PCR analysis, and many detection chemistries are available, including probe-based systems that provide assurance that the correct fragment is amplified. Primer sets that have shown *Salmonella* specificity in traditional PCR applications require evaluation in real-time PCR conditions to determine if these sets are specific and sensitive. Conventional PCR primers that have a strong tendency to form primer dimers may yield poor sensitivity results in a real-time assay. Since primer dimers are dsDNA, they will generate fluorescent signals (Higuchi *et al.* 1992) that yield false-positive results. If the primers form dimers, then at the lower target concentrations it may not be possible to distinguish between target fluorescent signals and primer dimer fluorescent signals by examining plots of relative fluorescence as a function of cycle number. In the absence of conventional PCR primers that yield adequate detection limits in real-time PCR, new primers can be designed or a probe-based system can be designed that yields fluorescence only in the presence of a specific dsDNA target.

Several real-time PCR assays have recently been developed for the detection of *Salmonella* (Hoorfar *et al.* 2000; Eyigor *et al.* 2002; Bhagwat 2004). Previous studies have shown comparisons of primers used in traditional PCR assays (Gooding and Choudary 1999; Malorny *et al.* 2003; Ziemer and Steadham 2003). The purpose of this work was to compare primer sets in a real-time PCR assay and to find a primer set that gives specific and sensitive detection of

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Salmonella enterica serovars associated with food-borne outbreaks from produce.

MATERIALS AND METHODS

Bacterial serovars and growth media

All bacteria used in this study are listed in Table 1. Bacteria were cultured on Luria-Bertani agar plates (LB, Difco/BBL, Sparks, MD, USA). Bacteria from an 18-h culture grown on LB plates were diluted in sterile water to an optical density of 0·2 at 600 nm. Serial dilutions were made in sterile water to obtain appropriate cell densities for PCR testing. Cell densities were quantified by plating 100 μl of the appropriate serial dilution on LB plates. The plates were incubated at 37°C overnight and colonies were counted.

Reagents and primers

Primer set sequences and references are shown in Table 2. All primer sets in Table 2, other than Sen, were shown to be specific for *Salmonella* in previously published research by testing with *Salmonella* and non-*Salmonella* target DNA. SYBR Green Master Mix (Applied Biosystems, Foster City, CA) reagent was used in initial experimentation with the Sal, INVA, ST, Fim, and iroB primer sets at primer concentrations of 500 nmol l^{-1}. However, further experimentation with low *Salm. enterica* cell concentrations indicated that there was a tendency for primer dimer formation. In an attempt to minimize this problem, SYBR Green Core Reagents (Applied Biosystems) were used to allow changes in the reagent concentrations. The final core reagent concentrations used in each 50 μl reaction were: 1X SYBR Green PCR Buffer, 3·0 mmol l^{-1} $MgCl_2$, 1·0 mmol l^{-1} dNTP mix with dUTP, and 1·25 U AmpliTaq Gold. In addition, 0·5 U AmpErase UNG enzyme (Applied Biosystems) was added to all Sen primer set reactions. Reduced concentrations of 250 nmol l^{-1} were tested with the Sal and INVA primer sets in sensitivity testing. All experiments with the Sen primer set were performed at primer concentrations of 250 nmol l^{-1} with the core reagents.

Mixtures of SYBR Green Master Mix or SYBR Green Core Reagents with the appropriate primer set were vortexed prior to transfer to the PCR tubes. The total volume of SYBR Green mix including primers was 30 μl (including water if necessary to adjust the volume to 30 μl). Twenty microlitres of the appropriate *Salm. enterica* serial dilution were then added to each tube to give a total

Table 1 Bacteria used for specificity testing

	Strain	Reference/source
Salm. enterica (serovar, serogroup)		
Saintpaul, B	55	SARB*
Schwarzengrund, B	96E01152C-TX-1	Inami and Moler (1999); CDHS†
Mbandaka, C_1	99A1670	CDHS
Newport, C_2	96E01153C-TX	Inami and Moler (1999); CDHS
Albany, C_3	96E01152C-TH	CDHS
Enteritidis, D_1	00A-2768	CDHS
Baildon, D_2	99A-23	CDHS
Meleagridis, E_1	96A7406	CDHS
Senftenberg, E_4	59	SARB
Rubislaw, F	54	SARB
Poona, G_1	00A3563	Barak *et al.* (2003); CDHS
Cubana, G_2	98A9878	Mohle-Boetani *et al.* (2001); CDHS
Havana, G_2	98A4399	Mohle-Boetani *et al.* (2001); CDHS
Saphra, I	97A3312	Mohle-Boetani *et al.* (1999); CDHS
Non-*Salmonella* species		
E. coli O137:H41	MW421	Wachtel *et al.* (2002)
Rahnella aquatilis	SPS2F10	Barak *et al.* (2002)
Pseudomonas putida	BM19	Barak *et al.* (2002)
Pantoea agglomerans	SPS2F1	Barak *et al.* (2002)
Erwinia chrysanthemi	3937	N. Perna‡
Klebsiella pneumonia	S48565.3	SFGH§

*SARB, *Salmonella* reference collection B.
†CDHS, California Department of Health and Human Services.
‡Nicole Perna, University of Wisconsin-Madison.
§SFGH, San Francisco General Hospital.

Table 2 Primer target, name, sequence, expected amplicon length and reference

Target fragment	Primer sets	Primer (5′ → 3′)	Product size (bp)	Reference
invA gene	Sal-3	TAT CGC CAC GTT CGG GCA A	275	Wang *et al.* (1997)
	Sal-4	TCG CAC CGT CAA AGG AAC C		
invA gene	INVA-1	ACA GTG CTC GTT TAC GAC CTG AAT	244	Chiu and Ou (1996)
	INVA-2	AGA CGA CTG GTA CTG ATC GAT AAT		
iroB gene	Primer 1	TGC GTA TTC TGT TTG TCG GTC C	606	Bäumler *et al.* (1997)
	Primer 2	TAC GTT CCC ACC ATT CTT CCC		
Random fragment of *Salm.* Typhimurium	ST11	GCC AAC CAT TGC TAA ATT GGC GCA	429	Soumet *et al.* (1999)
	ST15	GGT AGA AAT TCC CAG CGG GTA CTG G		
fimA gene	Fim1A	CCT TTC TCC ATC GTC CTG AA	85	Cohen *et al.* (1996)
	Fim2A	TGG TGT TAT CTG CCC GAC CA		
invA gene	Sen-1	TTT CAA TGG GAA CTC TGC	172	This work
	Sen-2	AAC GAC GAC CCT TCT TTT		

reaction volume of 50 μl. Negative control reactions used 20 μl of sterile water to make a total reaction volume of 50 μl.

Polymerase chain reactions were performed with a GeneAmp 5700 Sequence Detection System (Applied Biosystems) using the following thermal cycling conditions: 94°C for 5 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 7 min. The annealing temperature was lowered to 54°C for the Sen primer set due to its lower melting temperature. Prior to the 94°C hold for 5 min, an initial step of 50°C for 2 min was added to the Sen primer set protocol to activate the AmpErase UNG enzyme. After each PCR, a dissociation profile was generated by increasing the temperature from 60 to 90°C in increments of 0·4°C over 20 min, while measuring changes in fluorescence.

Gel analysis

In addition to dissociation curve analysis, PCR amplification fragments were run in 2% agarose gels in Tris–acetate–EDTA buffer to confirm fragment size. Following the PCR, 2 μl of 6X tracking dye (Promega, Madison, WI, USA) were mixed into the 50 μl reaction volume and 10 μl of this mixture were used for gel electrophoresis. A Hi-Lo DNA ladder (Minnesota Molecular, Minneapolis, MN, USA) was used to compare fragment sizes. Gels were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) or ethidium bromide for 10–20 min. The agarose gels were then photographed under u.v. excitation.

Primer design

Software (Primer Premier 5.0, PREMIER Biosoft International, Palo Alto, CA, USA) was used for assessment of the previously published primer sets and design of a new one. Given a particular target sequence and primer set, the software calculates the Gibbs free energy (ΔG) required for specific and nonspecific products to form, thereby allowing primers to be evaluated prior to experimentation. A large negative ΔG means that a reaction is energetically favourable and, therefore, indicates a tendency to form a dsDNA product. In addition to searching for potential primer dimers, the software can find locations outside of the target region where primers have a tendency to anneal, leading to nonspecific product formation called false priming. The *invA* gene from *Salm. enterica* serovar Typhimurium LT2 (accession number: AE008832, McClelland *et al.* 2001) was used as target DNA for software analysis of the Sal and INVA primer sets, and for designing a new primer set.

DNA sequencing

PCR products from the Sen primer set were sequenced to make certain that the target sequence was amplified. PCR products for sequencing were produced with the Sen primer set real-time PCR procedure described previously. DNA samples were purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Sequencing was performed by the Division of Biological Sciences DNA Sequencing Facility (University of California, Davis, CA, USA).

Experimental procedures

Each primer set was evaluated for its ability to amplify products from *Salm. enterica* serovars and from low initial template concentrations (sensitivity). Experimental conditions and procedures are summarized in Table 3. The Sal, INVA, iroB, ST, and Fim primer sets were tested in one experiment with the *Salm. enterica* serovars listed in Table 1 at 10^3CFU per reaction (rxn), and the Sen primer set was tested in one experiment with all bacteria shown in Table 1 at 10^3 CFU rxn^{-1}. Dissociation plot peaks and amplification fragments on agarose gels were used to verify that the

Table 3 Procedures and experimental conditions

Procedure	Primer set	[Primer] (nmol l^{-1})	PCR reagents*	Bacteria	[Bacteria] (CFU rxn^{-1})
Initial screening, traditional primers	Sal, INVA, iroB, Fim, ST	500	Master Mix	*Salm. enterica* serovars, Table 1	10^3
Sensitivity	Sal, INVA	250	Core Reagents	*Salm. enterica* Newport	10^6–10^3
Software analysis	Sal, INVA, Sen	–	–	–	–
Specificity	Sen	250	Core Reagents	All bacteria, Table 1	10^3
Sensitivity	Sen	250	Core Reagents	*Salm. enterica* Newport	10^6–10^0

*SYBR Green Master Mix or Core Reagents.

correct sequences were amplified. A primer set was considered for sensitivity testing if the initial results indicated single peaks in the dissociation plots, no peaks in the negative control dissociation plots and if agarose gel electrophoresis indicated that the correct size fragment was formed. Sensitivity testing involved three replicates per experiment using three separate experiments with each primer set. *Salm. enterica* Newport, an outbreak strain associated with produce, was used for sensitivity testing with levels ranging from 10^6 to 10^3 CFU rxn^{-1} for the Sal and INVA primer sets and approximately 10^6 to 10^0 CFU rxn^{-1} for the Sen primer set.

The detection limit of a particular primer set was determined by comparing the mean threshold cycle (C_T) value of the dilution with the mean C_T value of the negative control reactions. In the case of the Sal and INVA primer sets a one-tailed Student's *t*-test with $\alpha = 0{\cdot}05$ was performed to determine if there was a significant difference between the mean C_T values of the negative control reactions and the reactions containing the *Salm. enterica* Newport dilution of interest. Primer set detection limits were established by determining the lowest *Salm. enterica* Newport cell density mean C_T that was significantly different from the negative control.

RESULTS

Testing Sal, INVA, iroB, Fim, and ST primer sets using *Salm. enterica* serovars

The Sal, INVA, iroB, Fim and ST primer sets were tested with all *Salm. enterica* serovars shown in Table 1. Figure 1(a,b) show typical dissociation plots with each of the primer sets. The dissociation plots of the iroB and ST primer sets displayed multiple peaks with each serovar, while agarose gel electrophoresis showed a single band at the expected size in all cases. Without sequencing the PCR products, it was difficult to conclude if single or multiple products were formed with the iroB and ST primer sets. Furthermore, the iroB gel results produced faint bands and small dissociation peaks for *Salm. enterica* serovars Newport, Albany, Enteritidis and Baildon with significant amounts of primer dimer formation seen in these reactions. Thus, the iroB and ST primer sets were not used in the sensitivity experiments.

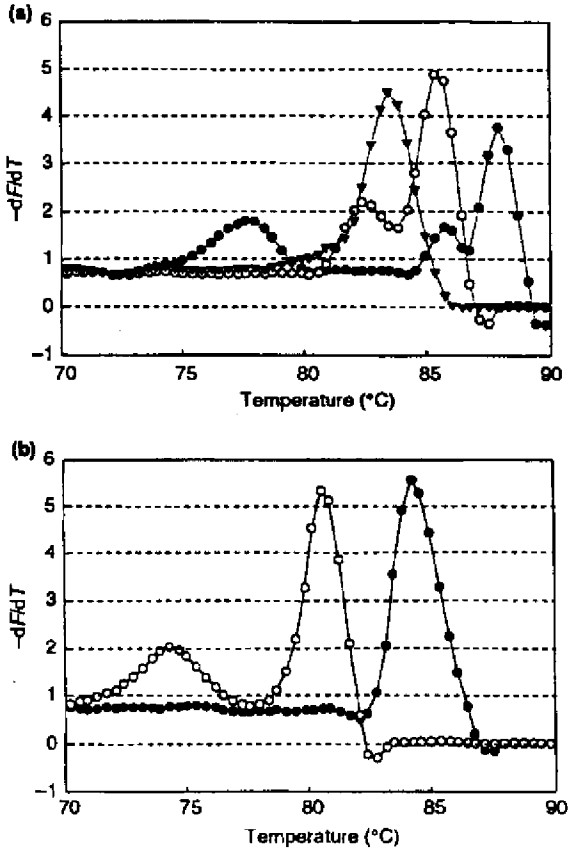

Fig. 1 Results of specificity testing using the iroB, ST, Fim, Sal, and INVA primer sets with *Salm. enterica* Schwarzengrund at a concentration of 6×10^4 CFU rxn^{-1}. Primer sets: (a) iroB (●), ST (○), Fim (▼); (b) Sal (●), INVA (○)

The Fim primer set produced a single 85 bp product and a single peak in the dissociation plot. However, primer dimers formed in the negative control reaction of the same size and position on the dissociation plot as the expected product. Testing this primer set at a reduced concentration of 250 nmol l^{-1} could help to reduce primer dimer formation. However, even if the size of the primer dimer seen in the negative control reaction was reduced, it may be difficult to interpret results from the *Salm. enterica* reactions because it will not be possible to tell if the fluorescent signal generated is from the target product, a primer dimer, or a combination of both. As it was not possible to distinguish between the *Salm. enterica* reactions and the negative control reactions in either dissociation analysis or gel electrophoresis, the Fim primer set was not used for further experimentation.

The INVA primer set results indicated that products of the correct size were formed with all *Salm. enterica* serovars tested. Figure 1(b) shows a large primary peak at 80°C along with a smaller secondary peak (which may have been a primer dimer) at a melting temperature of about 74°C. The larger peak in the dissociation analysis corresponded to the 244 bp product seen in the agarose gel (data not shown), as expected. As the primary product peak was clearly distinguishable from the smaller peak, the INVA primer set was used for sensitivity testing.

All *Salm. enterica* serovars tested with Sal were detected at 10^3 CFU rxn^{-1} with little to no primer dimer formation. The peak seen at 85°C in the dissociation plot of Fig. 1(b) corresponded to the expected 275 bp product seen in the gel analysis. The Sal primer set yielded the expected product for all *Salm. enterica* serovars and was therefore selected for sensitivity analysis.

Sensitivity of the Sal and INVA primer sets

In the case of both Sal and INVA primer sets, the dissociation plots showed that as the amount of initial template decreased, the height of the primer dimer peak increased and the height of the product peak decreased (data not shown). The detection limits of the Sal and INVA primer sets were approx. 3×10^3 and 3×10^4 CFU rxn^{-1}, respectively.

Primer analysis and design

As a lower detection limit was desired, software was used to compare the Sal and INVA primer sets with a new primer set design. Analysis of INVA and Sal indicated that these primer sets might form primer dimers, as indicated by the relatively large negative ΔG values associated with different primer dimer formations (data not shown).

A new primer set, Sen (*Salm. enterica*), also targeting the *invA* gene was designed and tested for *Salm. enterica* specificity using *Salm. enterica* and non-*Salmonella* isolates. The detection limit of Sen was also tested with *Salm. enterica* Newport. Table 2 gives the Sen primer set sequence. The software results indicated that only the reverse primer, Sen-2, has the potential for false priming on the *invA* gene (data not shown).

Sen primer set specificity and sensitivity

All *Salmonella* serovars shown in Table 1 formed a single product near the expected size, based on the real-time PCR dissociation plots and agarose gel electrophoresis results (data not shown). Sen primer set PCR products were sequenced in both directions and the product formed was the expected length of 172 bp. A BLAST analysis of the sequence results indicated that the correct sequence of bases was formed (accession number: AE008832, McClelland *et al.* 2001). None of the non-*Salmonella* bacteria crossed the real-time PCR threshold value, Rn = 0·5, over the course of 40 cycles (data not shown). Therefore, this primer set was considered specific for *Salm. enterica*.

Typical sensitivity results with the Sen primer set are shown in Fig. 2. Figure 2(a) shows that *Salm. enterica* Newport was detectable from 6×10^5 to 6×10^0 CFU rxn^{-1}, while the negative control reaction did not generate visible dsDNA. Additionally, as seen in Fig. 2b, no primer dimers were formed with the *Salm. enterica* Newport dilutions and all product peaks were found at a temperature of 82 ± 1°C.

The Sen sensitivity results indicate that the negative control reactions did not generate enough dsDNA to cross the relative fluorescence threshold value. As no negative control C_T values were available for comparison with *Salm. enterica* reaction C_T values, no statistical tests were performed. Detection limits of 80, 6 and 20 CFU rxn^{-1} were found in three separate runs, giving an average detection limit of 35 CFU rxn^{-1} with a standard deviation of 39 CFU rxn^{-1}.

DISCUSSION

In comparing the six primer sets for detection of *Salm. enterica* with real-time PCR, we chose to use pure cultures in sterile water as the first step in assay development. The formation of primer dimers in real-time PCR applications generates false-positive signals (Higuchi *et al.* 1992). These primer dimers ultimately lead to poor detection limits even without the presence of inhibiting substances found in field samples.

With varying degrees of amplification, all primer sets tested with the *Salm. enterica* serovars shown in Table 1 formed PCR products of the expected sizes at

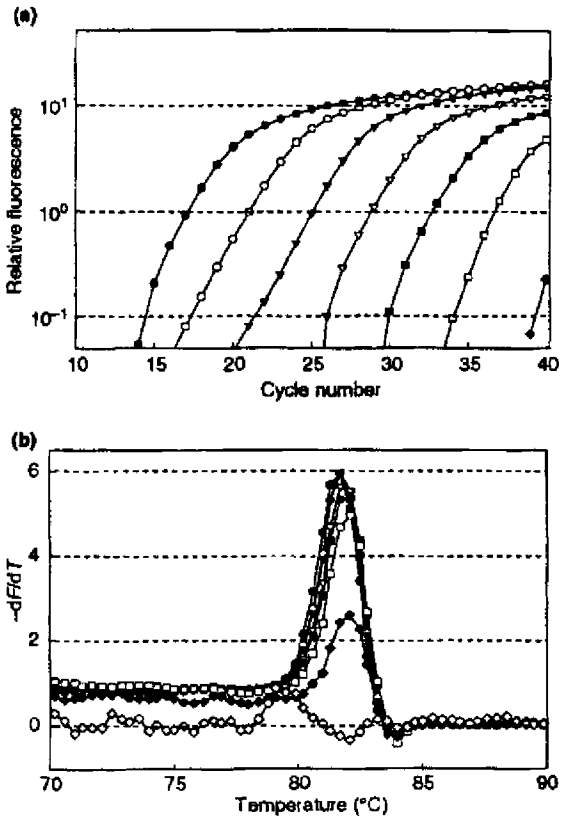

Fig. 2 Amplification and dissociation plots with the Sen primer set amplifying the DNA of *Salm. enterica* Newport. Cell concentrations: (a) 6×10^5 CFU rxn^{-1} (●), 6×10^4 CFU rxn^{-1} (○), 6×10^3 CFU rxn^{-1} (▼), 6×10^2 CFU rxn^{-1} (▽), 6×10^1 CFU rxn^{-1} (■), 6×10^0 CFU rxn^{-1} (□), 6×10^{-1} CFU rxn^{-1} (◆). Cell concentrations: (b) 6×10^5 CFU rxn^{-1} (●), 6×10^4 CFU rxn^{-1} (○), 6×10^3 CFU rxn^{-1} (▼), 6×10^2 CFU rxn^{-1} (▽), 6×10^1 CFU rxn^{-1} (■), 6×10^0 CFU rxn^{-1} (□), 6×10^{-1} CFU rxn^{-1} (◆), negative control (◇)

10^3 CFU rxn^{-1}. However, multiple peaks were seen in the dissociation plots of the ST and iroB primer sets with most of the *Salm. enterica* serovars tested, and this may have been the result of two products formed that were close to one another or a single product with a DNA sequence that generates a complex melting profile. Li *et al.* (2003) demonstrated that complex melting profiles can result from small single PCR products with SYBR Green dye. Their data indicate that regions of high A/T and G/C bases contribute to the shape of the melting profile of a product. A complex melting profile in the dissociation analysis of a PCR product that shows a single band on a gel requires sequencing to confirm that one product was actually formed and co-migrating bands were not present.

With the exception of one sample at 8 CFU rxn^{-1}, all dissociation plots for Sen primer products displayed single peaks larger than the background signal. The dissociation analyses confirmed that the correct melting temperature was found in each of the Sen primer set products generated.

Although extraction and enrichment procedures may improve the sensitivity of PCR, both steps are time consuming and labour intensive and, thus, were not used here to prepare the samples. Without an enrichment technique to increase levels of target DNA or an extraction process to purify DNA from a sample, the choice of primer set is critical in a real-time PCR application. At high template concentrations (10^6–10^5 CFU rxn^{-1}), both the Sal and INVA primer sets were consistently able to amplify the target fragment of interest. However, PCR with less template often generated primer dimers, ultimately yielding poor detection limits. The primer software was valuable in predicting the tendency for dimer formation and designing the Sen primer set which was ultimately the most sensitive primer set we tested. Using primers developed for traditional PCR may lower real-time assay sensitivity. Therefore, new primers are required specifically for real-time PCR.

The ultimate goal of this research is to build an automated biosensor for field use to screen for pathogens in the irrigation/wash water from fruits and vegetables. Jackson *et al.* (2003) started this work and developed components for such a biosensor. An ideal biosensor rapidly confirms the presence or absence of its target. While enrichment techniques have been used prior to PCR for sensitive detection of *Salmonella* (Eyigor *et al.* 2002; Bhagwat 2004), these procedures typically take hours and such practices are incompatible with the goal of rapid detection. In order to improve our detection limit, an automated concentration mechanism is under development for the purpose of increasing the cell densities of liquid test samples. DNA extraction steps may also improve PCR target detection, but not all steps in an extraction process are easily automated. Obtaining a specific and sensitive primer set for real-time PCR detection of *Salm. enterica* under ideal conditions was the first step in the development of an automated biosensor. The next step will be testing *Salm. enterica* detection performance in spiked water samples from produce operations.

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